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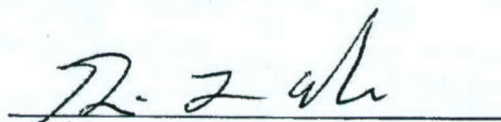
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Quorum Sensing: a Transcriptional Regulatory System Involved in the Pathogenicity of *Burkholderia mallei*

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Numerous gram-negative bacterial pathogens regulate virulence factor expression by using a cell density mechanism termed quorum sensing (QS). An *in silico* analysis of the *Burkholderia mallei* ATCC 23344 genome revealed that it encodes at least two *luxI* and four *luxR* homologues. Using mass spectrometry, we showed that wild-type *B. mallei* produces the signaling molecules *N*-octanoyl-homoserine lactone and *N*-decanoyl-homoserine lactone. To determine if QS is involved in the virulence of *B. mallei*, we generated mutations in each putative *luxIR* homologue and tested the pathogenicities of the derivative strains in aerosol BALB/c mouse and intraperitoneal hamster models. Disruption of the *B. mallei* QS alleles, especially in RJ16 (*bmaII*) and RJ17 (*bmaI3*), which are *luxI* mutants, significantly reduced virulence, as indicated by the survival of mice who were aerosolized with 10⁴ CFU (10 50% lethal doses [LD₅₀s]). For the *B. mallei* transcriptional regulator mutants (*luxR* homologues), mutation of the *bmaR5* allele resulted in the most pronounced decrease in virulence, with 100% of the challenged animals surviving a dose of 10 LD₅₀s. Using a Syrian hamster intraperitoneal model of infection, we determined the LD₅₀s for wild-type *B. mallei* and each QS mutant. An increase in the relative LD₅₀ was found for RJ16 (*bmaII*) (>967 CFU), RJ17 (*bmaI3*) (115 CFU), and RJ20 (*bmaR5*) (151 CFU) compared to wild-type *B. mallei* (<13 CFU). These findings demonstrate that *B. mallei* carries multiple *luxIR* homologues that either directly or indirectly regulate the biosynthesis of an essential virulence factor(s) that contributes to the pathogenicity of *B. mallei* *in vivo*.

Burkholderia mallei, the etiologic agent of glanders, is a gram-negative, oxidase-positive, nonmotile bacillus that is an obligate animal pathogen (4). The natural hosts for *B. mallei* are horses, donkeys, and mules (solipeds). Until the early 20th century and the development of motorized transportation, glanders was common throughout the world (4). After the implementation of quarantine strategies for imported animals, no naturally occurring human cases of glanders have been reported in the United States since the 1930s. Human glanders is uncommon now, occasionally occurring in individuals such as veterinarians, slaughterhouse workers, and laboratory scientists whose occupation exposes them to infection. In solipeds, two distinctive forms of glanders may arise, either an acute (observed with mules and donkeys) or a chronic (common in horses) form. Symptoms of acute glanders include weight loss, difficulty breathing, and an elevated temperature. In contrast, horses with chronic glanders may exhibit pulmonary and cutaneous (farcy) symptoms. Human acute glanders is characterized by fever and fatigue as well as inflammation of and nodule formation on the face and peripheral limbs (4). Chronic glanders in humans presents with swollen lymph nodes, ulcerating nodules in the alimentary and respiratory tracts, weight loss, and numerous subcutaneous abscesses (4). *B. mallei* can cause disease in a variety of animals, including mice, hamsters, ferrets, guinea pigs, and monkeys, in addition to solipeds and humans (9, 22).

Many gram-negative bacteria possess sophisticated communication systems that allow microorganisms to detect and respond, in a cell-density-dependent manner, to fluctuating environmental conditions at the transcriptional level. This ability to couple extracellular and intracellular signals, termed quorum sensing (QS), involves the synthesis and accumulation of *N*-acyl-homoserine lactones (AHLs) (6, 10, 13). AHL biosynthesis is enzymatically mediated by the LuxI family of proteins, which are *N*-acyl-homoserine lactone synthases (AHSs), and a single AHS may produce multiple AHLs with various acyl chain lengths and chemical modifications (12). Cytosolic LuxR proteins respond to AHLs in a concentration-dependent manner through binding of the membrane-permeable signal molecule (AHL). This AHL-protein interaction facilitates conformational changes and multimerization, which in turn induces or represses target gene expression (11). In animal and plant pathogens, this coordinated gene expression of alleles encoding proteins needed for virulence allows microorganisms to elicit an overwhelming attack before host cells can mount an effective defense (2, 7, 12, 17, 25, 30, 37).

Functional QS networks have recently been identified in *Burkholderia cepacia*, *Burkholderia vietnamiensis*, *Burkholderia thailandensis*, and *Burkholderia pseudomallei* (1, 3, 15, 20, 32, 34–36). Collectively, these *Burkholderia* QS networks have been shown to both positively and negatively regulate various cellular processes, including AHL and protease production, siderophore biosynthesis, biofilm formation, lipase and beta-hemolytic activities, swarming and twitching motilities, and substrate utilization (18, 20, 21, 32, 35, 36). Furthermore, disruption of these cell signaling systems has been shown to reduce the pathogenicity of *B. cepacia* and *Burkholderia*

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TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Description ^a	Source
Strains		
<i>Escherichia coli</i>		
SM10	Mobilizing strain, RP4 <i>tra</i> genes, Km ^r	29
TOP10	Used for cloning, gene expression, and blue-white screening	Invitrogen
RJ23	TOP10 containing pRUR6	This study
RJ24	TOP10 containing pRUR7	This study
<i>Agrobacterium tumefaciens</i>		
NTL4	Ti plasmidless derivative, nopaline chromosomal background	11
<i>Burkholderia mallei</i>		
ATCC 23344	Type strain genome sequence completed ^b	American Type Culture Collection
RJ16	ATCC 23344 <i>bmaI1</i> ::pRUI1, Gm ^r	This study
RJ17	ATCC 23344 <i>bmaI3</i> ::pRUI3, Gm ^r	This study
RJ18	ATCC 23344 <i>bmaR1</i> ::pRUR1, Gm ^r	This study
RJ19	ATCC 23344 <i>bmaR3</i> ::pRUR3, Gm ^r	This study
RJ20	ATCC 23344 <i>bmaR5</i> ::pRUR5, Gm ^r	This study
RJ21	RJ16 containing pRUR6	This study
RJ22	RJ17 containing pRUR7	This study
Plasmids		
pGSV3	Mobilizable suicide vector, Gm ^r	5
pCR2.1-TOPO	TA cloning vector, Km ^r Ap ^r	Invitrogen
pBHR1	Broad-host-range expression vector, Km ^r Cm ^r	MoBiTec
pRUI1	pGSV3 containing a 369-bp PCR product from the ATCC 23344 <i>bmaI1</i> gene	This study
pRUI3	pGSV3 containing a 398-bp PCR product from the ATCC 23344 <i>bmaI3</i> gene	This study
pRUR1	pGSV3 containing a 397-bp PCR product from the ATCC 23344 <i>bmaR1</i> gene	This study
pRUR3	pGSV3 containing a 402-bp PCR product from the ATCC 23344 <i>bmaR3</i> gene	This study
pRUR5	pGSV3 containing a 401-bp PCR product from the ATCC 23344 <i>bmaR5</i> gene	This study
pRUR6	pBHR1 containing the <i>bmaI1</i> gene	This study
pRUR7	pBHR1 containing the <i>bmaI3</i> gene	This study

^a r, resistance; Km, kanamycin; Gm, gentamicin; Ap, ampicillin; Cm, chloramphenicol. *bma* represents the QS genes present in *B. mallei*; *bmaI1* to *I3* depict AHL synthases (*luxI* genes); and *bmaR1*, *bmaR3*, and *bmaR5* indicate transcriptional regulators (*luxR* alleles).

^b The sequence is available at <http://www.tigr.org/>.

pseudomallei in murine and hamster models of infection (1, 31, 34, 36).

Considering that no effective vaccine is available against glanders as well as the risk of *B. mallei* weaponization, investigations focusing on vaccine development against this highly infectious *Burkholderia* species are essential. The objective of this study was to analyze the functional role between QS and the pathogenicity of *B. mallei*. Utilizing two animal models of infection, we clearly demonstrate that QS is involved in the pathogenicity of *B. mallei*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and cloning vectors used for this study are described in Table 1. *B. mallei* was cultured in Luria-Bertani (LB) broth or on plates containing 4% glycerol (LBG) (Sigma, St. Louis, Mo.). *Escherichia coli* strains containing recombinant clones were grown on LB plates or in broth containing 25 µg of kanamycin (Sigma)/ml and 50 µg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Sigma)/ml by using standard procedures (26). For AHL detection, *Agrobacterium tumefaciens* NTL4 was cultured in AT minimal medium at 30°C (11).

Cloning of *B. mallei* QS genes, mutant construction, gene disruption, and mutant confirmation. PCR primers for disruption cassettes were made by using the *B. mallei* ATCC 23344 sequences (The Institute for Genomic Research) that were confirmed in silico to carry putative *luxI* genes. Genomic DNA for PCR amplification was purified by using a MasterPure DNA purification kit according to the manufacturer's instructions (Epicentre Technologies, Madison, Wis.). Internal gene fragments were PCR amplified with the primer pairs listed in Table 2 under the following conditions: 1 cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and a final 7-min extension at 72°C. Site-specific integration was confirmed by using the cycling conditions described above, with a 5-min extension time, by use of the gene-specific PCR primer sets

TABLE 2. Primers used for this investigation

Primer target	Primer sequence (5'-3') ^c
Internal gene primers^a	
<i>bmaI1</i>	F CCGCGACGACGACGGGGAAATC R TCGATCCAGCACGCGACGACCAT
<i>bmaI3</i>	F TCGCGGGCCGATTGAACGAAGTGC R GAGCGACGCGGCCACCGTGAGCAC
<i>bmaR1</i>	F CGGCTTCGAATATTGCTGCTATGG R GAGAAAACGGCTCATCAGCGAGTG
<i>bmaR3</i>	F AGACGTCGTCTCGCTGCACTATCC R ACCACGTGAGGCACATCTGTTCC
<i>bmaR4</i>	F GCGCTTCGACAGATGAAACACGAC R GCTCATCTGGCAGCAGACCTCTA
<i>bmaR5</i>	F CGCGTGCGGTGGCCGCTGTCCA R CCGCGCTCCGGGTCCGCCATCAG
Mutant confirmation primers^b	
<i>bmaI1</i>	F GCGCGAAACACGAGTCCCTGTCT R TTTTCCTCGAACGTTGCGGATTGA
<i>bmaI3</i>	F CGGCGGTCCGGTTAGAGAGAACG R CGCCTTCGTGTCGCGCAACAGC
<i>bmaR1</i>	F GAAGCGGAACCGTTGATGGAGTGA R AGCGTGAAGCTGCTGGAGAACGAA
<i>bmaR3</i>	F GCGACACGAAGGCGCGCGGATAC R GTGCGGGGTGCTGCTCGGGAGAAA
<i>bmaR4</i>	F AAAGTGCCTGCACCTACGCTTTTG R CTTGAGCTGGCGGTTCTATGTTTC
<i>bmaR5</i>	F AAACCGCATAAGCACAATCAATCA R GAGCTTCAGGATCGGTTCTTCA

^a Primers used for the construction of gene disruption cassettes.

^b Primers utilized to confirm site-specific integration of the suicide vector.

^c F, forward primer; R, reverse primer.

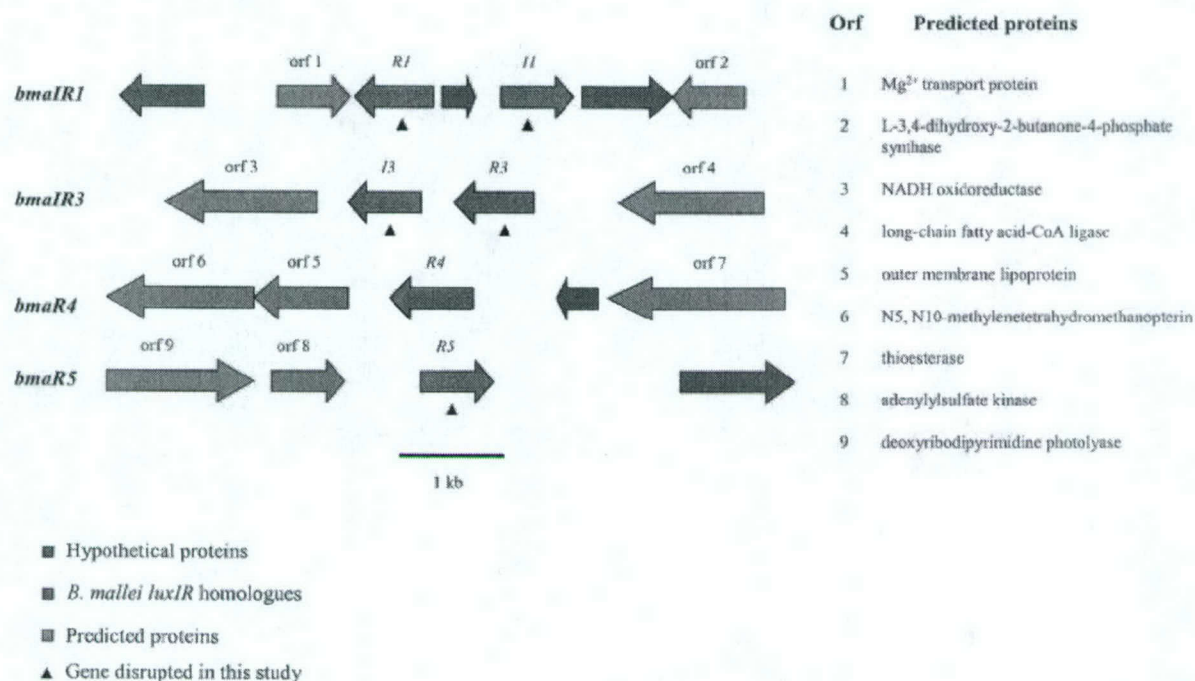


FIG. 1. Genetic organization of *B. mallei* QS loci. Approximately 6-kb segments that were confirmed in silico to carry putative *luxIR* homologues were used for structural analysis and ORF prediction. Genes identified in *B. mallei* ATCC 23344 are represented by *bma*, and triangles (▲) denote the mutated alleles analyzed in this study. Attempts to create mutations in *B. mallei* *bmaR4* were unsuccessful.

listed in Table 2. PCR amplification was performed by using an Epicentre FailSafe kit with buffer J (Epicentre Technologies). Reactions were analyzed by standard methods, and the products were subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.). Ligation products were transformed into One Shot chemically competent *E. coli* TOP10 cells and then screened accordingly (26). Mutant construction was performed as previously described (33). For gene expression in *E. coli* TOP10 cells, the *B. mallei* *luxI* genes were PCR amplified as described above, cloned into pCR2.1-TOPO, and chemically transformed into *E. coli* TOP10. Plasmid purification was performed by using a QIAprep Spin miniprep kit (Qiagen, Valencia, Calif.), and the resulting clones were digested with EcoRI (New England Biolabs, Beverly, Mass.) by standard methods (26). Digestion reactions were separated in a 1% agarose gel, and the bands were excised by use of a QIAquick gel extraction kit (Qiagen). Gel-purified amplicons were ligated into EcoRI-digested pBHRI by using a Fast-Link DNA ligation kit (Epicentre) and were chemically transformed into *E. coli* TOP10. Recombinant clones were screened for AHL biosynthesis (cross-streaking) by using the *A. tumefaciens* NTL4 bioreporter strain.

MS analysis of culture extracts. AHLs were extracted from culture supernatants with ethyl acetate (Sigma), which was then evaporated, and the extracts were resuspended in 1 ml of acetonitrile (Sigma) (28). Aliquots (150 μ l) were dried under a steady stream of dry nitrogen, reconstituted in 100 μ l of 50% acetonitrile, and passed through a 0.45- μ m-pore-size nylon filter. Approximately 500 nl of each extract was injected onto a CAPLC capillary liquid chromatograph (Waters Corporation, Milford, Mass.) fitted with an Aquasil C₁₈ high-performance liquid chromatography column (10 cm by 75 μ m) (New Objective, Woburn, Mass.) operating at a flow rate of 500 nl/min. A gradient elution was employed starting at 100% buffer A (2% acetonitrile–0.1% formic acid) and ending at 100% buffer B (80% acetonitrile–0.1% formic acid) in 30 min. A voltage of 2.1 kV was applied to the column effluent entering the nanoelectrospray source attached to a Q-TOF-2 mass spectrometer (Micromass, Beverly, Mass.). The source temperature was 125°C, and a cone voltage of 18 V was applied. Argon (10 Pa of nominal pressure) was used as the collision gas, with an energy setting of 15 V. The results obtained by mass spectrometry (MS) (scanning from *m/z* 160 to 330 in 1.5 s) were acquired by the use of data-directed analysis software (Waters Corporation). Ions meeting selected intensity and charge state criteria were further characterized by MS/MS. Precursor ions yielding a fragmentation ion at *m/z* 102, representing the lactone ring of AHL signaling molecules, were recorded, and the (*M* + *H*)⁺ values were determined.

Fragmentation ions of MS/MS spectra containing an ion at *m/z* 102 were compared to the fragmentation mass spectra of the corresponding AHL standard when possible. If a precursor ion with an (*M* + *H*)⁺ value that was not equal to any of the AHL standards yielded an MS/MS spectrum containing an ion at *m/z* 102, the mass spectra were further analyzed for the presence of ions that are characteristic of acyl side chains containing substitutions that lose a water molecule(s) after collision-induced dissociation.

Aerosol challenge, LD₅₀ analysis, and IgG titers. An inhalational challenge of female BALB/c mice, an analysis of the 50% lethal dose (LD₅₀), and measurements of immunoglobulin G (IgG) titers were performed as previously described (19, 24, 33, 38). Briefly, for aerosol exposures, wild-type *B. mallei* and each QS mutant were inoculated (100 μ l from a 3-ml overnight culture) into 10 ml of LBG broth and cultured with aeration (250 rpm) for 18 h at 37°C. Aerosolization (10 mice for each bacterial strain) was performed by nebulizing the entire 10-ml overnight culture (stationary phase), which delivered approximately 10 LD₅₀s.

RESULTS

Structural analysis and ClustalW nucleotide alignments of *B. mallei* QS alleles. Using the *B. cepacia* CepIR and *Pseudomonas aeruginosa* LasIR and RhlIR proteins, we searched the *B. mallei* ATCC 23344 genome (The Institute for Genomic Research) in silico for putative LuxIR homologues. This in silico analysis, which was confirmed with PCR amplification (data not shown), indicated that *B. mallei* carries at least four *luxR* and two *luxI* homologues (Fig. 1). The structural organization of *B. mallei* *luxIR* and the surrounding genes is depicted in Fig. 1. The results of Blastx homology searches for each *B. mallei* QS allele are summarized in Table 3, and ClustalW nucleotide sequence alignments are described below.

Briefly, the *bmaIR1* loci are divergently transcribed and are separated by a GeneMark (http://opal.biology.gatech.edu/GeneMark/gmhmm2_prok.cgi)-predicted 444-bp open reading frame (ORF) with no significant similarity to proteins in

TABLE 3. Blastx searches using the *B. mallei* QS proteins

Protein ^a	% Identity	% Similarity	Homologous protein ^b	Protein ID ^c
BamI1	99	99	<i>B. pseudomallei</i> BpsI AHL synthase	AAQ901683
BamI3	40	54	<i>Burkholderia multivorans</i> CepI AHL synthase	AF330013.1
BamR1	100	100	<i>B. pseudomallei</i> BpsR AHL receptor	AAR88244
BamR3	44	61	<i>Burkholderia fungorum</i> hypothetical protein	ZP_00030469.1
BamR4	33	47	<i>Pseudomonas putida</i> PpuR transcriptional regulator	AAM75413.1
BamR5	51	67	<i>Ralstonia solanacearum</i> transcriptional activator	NP_522339.1

^a Quorum-sensing proteins found in *B. mallei*.^b Corresponding species containing similar LuxIR proteins to those of *B. mallei*.^c GenBank protein accession numbers.

the National Center for Biotechnology Information databases (Fig. 1). In contrast, the *bmaIR3* alleles are genetically linked but are not disrupted by an intergenic sequence and are transcribed in the same direction (Fig. 1). Interestingly, a Blastx analysis of the 5' and 3' (3 kb on each side) regions flanking the *bpmR4* and *bpmR5* loci failed to recover any putative *luxI* genes, suggesting that these QS alleles are orphaned for a putative LuxI protein (Fig. 1).

Amino acid alignments (ClustalW) between the *B. mallei* BmaI1 and BmaI3 proteins, *B. cepacia* CepI, *P. aeruginosa* LasI and RhlI, *B. pseudomallei* BpsI, and the *B. pseudomallei* DD503 BpmI2 and BpmI3 (34) proteins revealed the presence of the 10 invariant amino acids (between residues 24 and 109) that are commonly found in LuxI proteins (23; also data not shown). Similarly, an alignment of the *B. mallei* LuxR proteins with *P. aeruginosa* RhlR and LasR, *B. cepacia* CepR, *B. pseudomallei* BpsR, and the *B. pseudomallei* DD503 BpmR2 to -5 transcriptional regulators identified six of the seven invariant amino acids that are found in LuxR proteins (8; also data not shown).

Detection and characterization of AHLs produced by wild-type *B. mallei*. To determine the AHL moieties that are synthesized by wild-type *B. mallei* and each *luxI* QS mutant, we performed an MS analysis of crude culture extracts. *B. mallei* produced both *N*-octanoyl-homoserine lactone (C_8 -HSL) and *N*-decanoyl-homoserine lactone (C_{10} -HSL), which was confirmed by an analysis of synthetic AHL standards (Table 4). In culture supernatants of RJ16, which contains a disruption in the *bmaI1* AHS locus, the signaling molecules C_8 -HSL, C_{10} -HSL, and *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3-hydroxy- C_8 -HSL) were identified (Table 4). In contrast to that for wild-type *B. mallei* and RJ16 (*bmaI1* mutant), the only AHL moiety identified in RJ17 (*bmaI3* mutant) culture supernatants was C_8 -HSL (Table 4). Disruption of the *bmaI1* gene had no effect on AHL biosynthesis, and in fact, supernatants of RJ16 contained an additional AHL (3-hydroxy- C_8 -HSL) that was not detected in wild-type *B. mallei* (Table 4). Likewise, culture extracts of RJ17 (*bmaI3* mutant) contained C_8 -HSL (Table 4). To address these discrepancies, we cloned the *bmaI1* and *bmaI3* genes into the broad-host-range expression vector pBHR1, transformed them into *E. coli*, and monitored the AHL biosynthesis profiles as described in Materials and Methods. RJ23 (expresses the *bmaI1* gene) supernatants contained C_8 -HSL, C_{10} -HSL, and 3-hydroxy- C_8 -HSL, while extracts from overnight cultures of RJ24 (heterologously expresses the *bmaI3* gene) contained C_8 -HSL, C_{10} -HSL, and *N*-(3-hydroxy-decanoyl)-L-homoserine lactone (3-hydroxy- C_{10} -HSL) (Table

4). All AHLs identified by MS in this investigation produced a fragment ion at *m/z* 102, which is characteristic of the lactone ring bound to the acyl side chain of AHLs. AHL standards for the hydroxy-substituted signaling molecules identified in this work (3-hydroxy- C_8 -HSL and 3-hydroxy- C_{10} -HSL) were not analyzed, but the MS profiles matched spectra from a previous study (28). A summary of the relevant fragmentation ions is shown in Table 4.

Disruption of the *B. mallei* QS system reduces virulence in an aerosol BALB/c mouse model. To analyze the course of acute infection for wild-type *B. mallei* and each QS mutant, we monitored animal survival after bacterial exposure for 39 days postexposure (p.e.) (Fig. 2). Groups of 10 female BALB/c mice were challenged with 10^4 CFU (10 LD₅₀s) of *B. mallei* and each QS mutant. Deaths for the group exposed to wild-type *B. mallei* began on day 5, and the remaining mice succumbed by 6 days p.e. (Fig. 2). Surprisingly, 100% survival at 39 days p.e. was observed for the experimental groups that were aerosolized with RJ16 (*bmaI1* mutant), RJ17 (*bmaI3* mutant), and RJ20 (*bmaR5* mutant) (Fig. 2). Deaths for mice challenged with RJ18 (*bmaR1* mutant) and RJ19 (*bmaR3* mutant) began on days 22 and 7, respectively, and continued over the 39-day course of analysis (Fig. 2). Although they were chronically infected (with splenic and hepatic abscesses, animal huddling, and fur ruffling), seven and four animals survived an aerosol challenge with RJ18 (*bmaR1* mutant) and RJ19 (*bmaR3* mutant), respectively, at 39 days p.e. (Fig. 2), in contrast to mice receiving wild-type *B. mallei*.

LD₅₀ determination, IgG titers, and vaccine efficacy. To further assess the correlation between QS and the pathogenicity of *B. mallei*, we employed an acute hamster model of glan-

TABLE 4. AHL profiles of *B. mallei* and each *luxI* QS mutant

AHL molecule ^a	Signature peaks (<i>m/z</i> values) ^b	Bacterial strains expressing molecule ^c
C_8 -HSL	228, 127, 109, 102	ATCC 23344, RJ16 (<i>bmaI1</i>), RJ17 (<i>bmaI3</i>), RJ23, RJ24
C_{10} -HSL	256, 155, 137, 102	ATCC 23344, RJ16 (<i>bmaI1</i>), RJ23, RJ24
3-Hydroxy- C_8 -HSL	244, 125, 102, 97	RJ16 (<i>bmaI1</i>), RJ23
3-Hydroxy- C_{10} -HSL	272, 153, 135, 102	RJ24

^a *N*-Acyl-homoserine lactones synthesized by *B. mallei* and each QS mutant.^b Signature peaks from mass spectrometry analysis of overnight culture extracts.^c Wild-type *B. mallei* is represented by ATCC 23344, and *bma* depicts the *luxI* homologues. RJ23 (*bmaI1*) and RJ24 (*bmaI3*) are *E. coli* strains expressing the *bmaI1* and *bmaI3* genes.

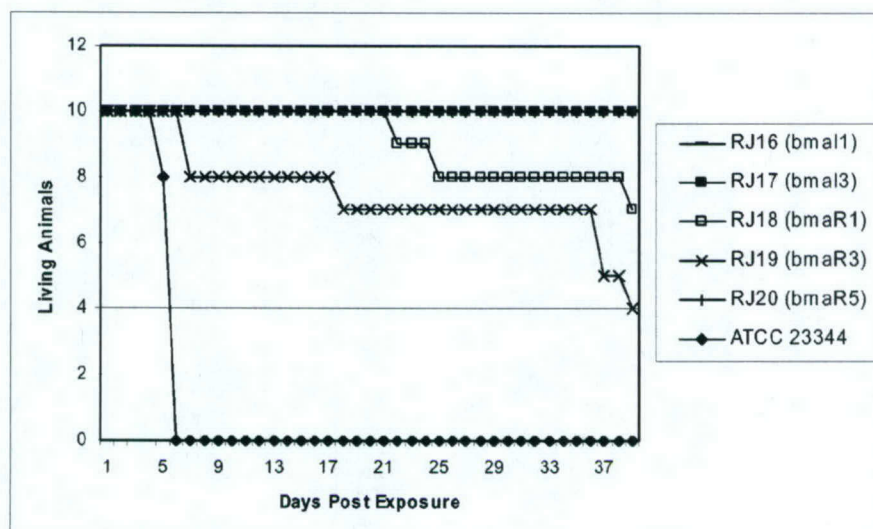


FIG. 2. Time to death of BALB/c mice who were aerosolized with *B. mallei* and each QS mutant. The survival patterns of animals who were challenged with wild-type *B. mallei* ATCC 23344 and the QS mutants after aerosol exposure are shown. A targeted dose of 10^4 CFU (10 LD₅₀s) was delivered, and animal mortality was monitored for 39 days. Disrupted *B. mallei luxIR* homologues are denoted by *bmaI* or *bmaR*.

ders. The relative LD₅₀ for wild-type *B. mallei* at 4 days p.e. was <13 CFU, whereas individual mutagenesis of the *B. mallei* QS genes increased the LD₅₀ up to approximately 100-fold (Table 5). Due to the sensitivity of Syrian hamsters to *B. mallei*, we performed complementation studies of the *luxI* mutants with this animal model. As expected, a reduction in the LD₅₀ occurred by heterologous expression of *bmaI1* and *bmaI3* in RJ16 and RJ17, respectively (Table 5).

In addition to determining the time to death for aerosolized BALB/c mice and determination of LD₅₀s in hamsters, we determined the IgG titers (in BALB/c mice) against *B. mallei* and each QS mutant, as previously described (38). Seropositive reactions, expressed as reciprocals of the highest dilutions producing positive results, were obtained for each of the *B. mallei* QS mutants in addition to the *B. mallei* positive control. The IgG titers for each strain are reported in Table 5.

For determinations of whether the *B. mallei* QS mutants provided protection against a challenge with wild-type *B. mallei*, experimental groups that were initially aerosolized with RJ16 (*bmaI1* mutant), RJ17 (*bmaI3* mutant), and RJ20 (*bmaR5* mutant) were rechallenged at 14 and 27 days p.e. and then aerosolized at 36 days p.e. with 10 LD₅₀s of wild-type *B. mallei*. Only pre-exposure to RJ17 (*bmaI3* mutant) conferred partial protection (3 of 10 animals) to a challenge with *B. mallei*, with mice surviving 11 days post-aerosolization, compared to unimmunized animals exposed to wild-type *B. mallei*, who died by 6 days p.e. (Fig. 2).

DISCUSSION

This investigation analyzed the role of QS in the pathogenicity of *B. mallei* in vivo by using aerosol BALB/c mouse and Syrian hamster models. In gram-negative bacteria, QS represents a complex mechanism for gene regulation through the synthesis and recognition of AHL signaling molecules. For human and plant pathogens, AHL-based communication sys-

tems allow a microbial community to strategically induce or repress expression of genes, specifically alleles encoding putative virulence factors, in response to environmental stimuli. Several reports have identified functional QS systems in various *Burkholderia* species and have shown that these bacterial communication networks both positively and negatively regulate numerous extracellular virulence factors in addition to contributing to animal pathogenicity (*B. cepacia*) (1, 3, 20, 21, 31, 34, 36).

The *B. mallei* QS system is extremely complex and is comprised of multiple *luxIR* homologues (Fig. 1). Our genome analysis in silico indicated that the *B. pseudomallei* and *B. mallei* QS networks are genetically similar, and as with *B. pseudomallei*, *B. mallei* does not encode a putative LuxS system (Fig. 1) (34). While our findings were under review, Valade et al. characterized two of the eight *luxIR* genes carried by *B. pseudomallei* (36). The *pmlIR* QS genes reported by Valade et al. correspond to the *B. mallei bmaIR1* QS alleles characterized in

TABLE 5. Bacterial LD₅₀s for hamsters and IgG antibody titer determination

Strain	LD ₅₀ (CFU) for Syrian hamsters ^a	IgG titer ^b
Wild-type <i>B. mallei</i>	<13	
RJ16 (<i>bmaI1</i>)	>967	400
RJ17 (<i>bmaI3</i>)	115	100
RJ18 (<i>bmaR1</i>)	17	200
RJ19 (<i>bmaR3</i>)	98	400
RJ20 (<i>bmaR5</i>)	151	100
RJ21	135	
RJ22	51	
Positive control		12,800

^a For LD₅₀ determination, male Syrian hamsters (five for each dose) were challenged with 10^1 , 10^2 , and 10^3 CFU of *B. mallei* and each QS mutant.

^b The *B. mallei* positive control was an irradiated culture aliquot; IgG titers were determined at 21 days p.e.

this work (36). Interestingly, our in silico and in vitro (PCRs of internal gene amplicons) analyses indicated that *B. mallei* does not carry two of the *luxIR* pairs (*bpmIR2*) that were identified in the *B. pseudomallei* DD503 genome (34). Although they are preliminary, and considering that *B. mallei* is a pathoadaptive obligate mammalian pathogen as well as proposed to be a clone of *B. pseudomallei* (14), these findings suggest that these QS alleles are not required for the in vivo pathogenicity of *B. mallei*. This hypothesis is further supported by the observation that the *B. mallei* ATCC 23344 genome is 1.5 Mb smaller than the *B. pseudomallei* K96243 chromosomes (data not shown). Through the evolution and divergence of *B. mallei* from *B. pseudomallei*, we hypothesize that *B. mallei* has undergone genomic modifications (i.e., insertion sequence-mediated deletions) that have resulted in the loss of the additional *luxIR* (*bpmIR2*) pair that is carried by *B. pseudomallei* and *Burkholderia thailandensis* (34, 35).

It has been proposed that microbial species carrying multiple *luxIR* genes obtain these alleles through horizontal gene transfer (16). In some instances, these horizontally acquired segments of nucleic acid deviate in G+C content compared to the recipient host. The relative G+C ratio of the ORFs carrying the putative *B. mallei luxIR* homologues as well as the flanking genes is consistent with the overall G+C content of the *B. mallei* genome, suggesting that these QS alleles have been present throughout the evolution of this highly infectious *Burkholderia* species (data not shown).

Our initial approach for AHL detection and characterization, which had limited success, incorporated thin-layer chromatography overlays with the bioreporter strain *A. tumefaciens* NTL4. To circumvent these limitations, we performed MS with culture extracts of *B. mallei* and each *luxI* mutant. In supernatants from *B. mallei*, the signaling molecules C₈-HSL and C₁₀-HSL were detected (Table 4). As with *B. mallei*, it was recently shown that *B. pseudomallei* 008 produces C₁₀-HSL via the PmlI protein (36). Similar to *B. mallei*, with the exception of *N*-(3-oxotetradecanoyl)-L-homoserine lactone, *B. pseudomallei* DD503 synthesizes C₈-HSL, 3-hydroxy-C₈-HSL, C₁₀-HSL, and 3-hydroxy-C₁₀-HSL (34). Furthermore, it has been shown that culture extracts from *B. thailandensis* DW503, a closely related *Burkholderia* species to *B. mallei* and *B. pseudomallei*, contain the signaling molecules *N*-hexanoyl-homoserine lactone, C₈-HSL, and C₁₀-HSL (35). Surprisingly, disruption of the *B. mallei luxI* homologues had a marginal effect on AHL biosynthesis, and in fact, mutagenesis of these genes resulted in the detection of signaling molecules that were not identified in wild-type *B. mallei* supernatants (Table 4). There are multiple scenarios that may have contributed to these observations: (i) the *B. mallei* genome may encode an additional LuxI protein(s), (ii) the *B. mallei* LuxI proteins may interact with multiple acyl-acyl carrier proteins (i.e., QS is involved in cellular metabolism), and (iii) the BmaI and BmaI3 proteins may synthesize overlapping signaling molecules. With regard to the hypothesis that QS in *B. mallei* may be involved in carbon metabolism (i.e., it may affect the biosynthesis of AHL precursors), it has been shown at the transcriptional level that QS in *P. aeruginosa* both positively and negatively regulates numerous enzymes that are involved in carbon metabolism (27, 39). Likewise, mutagenesis of the *B. thailandensis* QS system and enzymatic cleavage of the AHLs produced by this closely re-

lated *Burkholderia* species also affect substrate utilization (32, 35). To determine if the BmaI and BmaI3 proteins produce overlapping signaling molecules, each *B. mallei luxI* homologue was heterologously expressed in *E. coli*, and the AHL profiles were monitored. Table 4 clearly demonstrates that with the exception of 3-hydroxy-C₈-HSL (unique to RJ23) and 3-hydroxy-C₁₀-HSL (found only in RJ24 extracts), the BmaI and BmaI3 proteins, when expressed in *E. coli*, produce structurally similar AHLs, which may account for the AHL profiles observed for the *B. mallei luxI* mutants. However, before any definitive conclusions with regard to AHL biosynthesis can be made, it will be necessary to construct multideletion *B. mallei luxI* strains. Despite these fluctuations in AHL accumulation following mutagenesis of the *B. mallei luxI* homologues, definitive phenotypes for the *bmaI1* (RJ16) and *bmaI3* (RJ17) mutants were confirmed by the use of two independent animal models, indicating that AHL biosynthesis plays an essential role, either directly or indirectly, in the virulence of *B. mallei*. As proposed for *B. pseudomallei* DD503, it is possible that the timing of biosynthesis and the concentration of the *B. mallei* signaling molecules are important for in vivo pathogenicity.

It was recently demonstrated by the use of murine models of infection that QS is involved in the pathogenicity of *B. cepacia* and *B. pseudomallei* (1, 31, 34, 36). Mutagenesis of the *B. mallei* QS alleles caused a significant reduction in animal mortality compared to the mortality of mice aerosolized with wild-type *B. mallei* (Fig. 2). The most notable decrease in pathogenicity was observed for strains containing disruptions in the *bmaI1* (RJ16) and *bmaI3* (RJ17) *luxI* homologues (Fig. 2). Additionally, inactivation of the *bmaR5* (RJ20) gene resulted in a drastic reduction in animal mortality compared to that with wild-type *B. mallei* (Fig. 2). In fact, 100% of the animals (10 for each group) that were exposed to RJ16 (*bmaI1* mutant), RJ17 (*bmaI3* mutant), and RJ20 (*bmaR5* mutant) survived an aerosol challenge of 10⁴ CFU, representing 10 LD₅₀s (Fig. 2). As with the aerosol BALB/c model, a reduction in virulence was also observed for several of the *B. mallei* QS mutants in Syrian hamsters (Table 5). Similar to the BALB/c aerosolization results, although to a lesser degree, RJ16 (*bmaI1* mutant), RJ17 (*bmaI3* mutant), RJ19 (*bmaR2* mutant), and RJ20 (*bmaR5* mutant) demonstrated the largest reductions in pathogenicity compared to wild-type *B. mallei* (Table 5). Interestingly, 70% of the mice that were exposed to RJ18 (*bmaR1* mutant) survived the challenge, whereas in hamsters RJ18 exhibited an LD₅₀ similar to that of wild-type *B. mallei* (Fig. 2 and Table 5). These findings suggest that the *B. mallei* QS system may regulate unidentified host-specific virulence factors that are needed for mouse versus hamster pathogenicity.

For *B. cepacia* and *B. pseudomallei*, QS has been shown to both negatively and positively regulate the biosynthesis of potential extracellular virulence factors (1, 20, 21, 36). Recently, we found that QS in *B. thailandensis* DW503, a species that is closely related to *B. mallei* and *B. pseudomallei*, both positively and negatively regulates lipase and beta-hemolytic activities, swarming and twitching motilities, and carbon metabolism (32, 35). With the exception of swarming and twitching motilities (*B. mallei* is nonmotile), *B. mallei* and each QS mutant were tested for defects in beta-hemolytic, protease, lipase, and phospholipase C activities. It should be noted that *B. mallei* as well as *B. thailandensis* are normally nonhemolytic; however, mu-

tagenesis of the *B. thailandensis luxIR* genes resulted in enhanced β -hemolysis of sheep erythrocytes (35). Surprisingly, and consistent with the case for mutagenesis of the *B. pseudomallei* DD503 QS network, the phenotypes of the parental and mutant strains of *B. mallei* were identical (data not shown). These results suggest that QS in *B. mallei* does not regulate a factor(s) that contributes to beta-hemolytic, lipase, protease, and phospholipase C activities.

For *B. mallei*, the only definitive virulence determinants that have been shown to be required for pathogenicity are an extracellular capsule and type III secretion (5, 33). A transmission electron microscopy analysis of capsule biosynthesis in each *B. mallei* QS mutant indicated that capsule production was not affected (data not shown). We are currently using whole-genome DNA microarrays to determine if QS affects the transcription of the *B. mallei* type III secretion operon. The *B. mallei* QS system represents one of the most complex intraspecies communication systems identified for obligate mammalian pathogens. These findings for two animal models of infection clearly demonstrate that QS plays an essential role in the in vivo pathogenicity of *B. mallei*. Further studies, utilizing whole-genome DNA microarrays, will be needed to identify the virulence factor(s) regulated by this intricate bacterial cell signaling network.

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REFERENCES

- Adam Baldwin, P. A. S., J. Parkhill, and E. Mahenthalingam. 2004. The *Burkholderia cepacia* epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia cenocepacia*. *Infect. Immun.* **72**:1537-1547.
- Camara, M., P. Williams, and A. Hardman. 2002. Controlling infection by tuning in and turning down the volume of bacterial small-talk. *Lancet Infect. Dis.* **2**:667-676.
- Conway, B. A., and E. P. Greenberg. 2002. Quorum-sensing signals and quorum-sensing genes in *Burkholderia vietnamiensis*. *J. Bacteriol.* **184**:1187-1191.
- DeShazer, D., and D. Waag. Glanders: new insights into an old disease. In L. Lindler, F. Lebeda, and G. W. Korch (ed.), *Biological weapons defense: infectious diseases and counterbioterrorism*, in press. The Humana Press Inc., Totowa, N.J.
- DeShazer, D., D. M. Waag, D. L. Fritz, and D. E. Woods. 2001. Identification of a *Burkholderia mallei* polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. *Microb. Pathog.* **30**:253-269.
- Donabedian, H. 2003. Quorum sensing and its relevance to infectious diseases. *J. Infect.* **46**:207-214.
- Dunn, A. K., and J. Handelsman. 2002. Toward an understanding of microbial communities through analysis of communication networks. *Antonie Leeuwenhoek* **81**:565-574.
- Flavier, A. B., L. M. Ganova-Raeva, M. A. Schell, and T. P. Denny. 1997. Hierarchical autoinduction in *Ralstonia solanacearum*: control of acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. *J. Bacteriol.* **179**:7089-7097.
- Fritz, D. L., P. Vogel, D. R. Brown, D. Deshazer, and D. M. Waag. 2000. Mouse model of sublethal and lethal intraperitoneal glanders (*Burkholderia mallei*). *Vet. Pathol.* **37**:626-636.
- Fuqua, C., M. R. Parsek, and E. P. Greenberg. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* **35**:439-468.
- Fuqua, C., and S. C. Winans. 1996. Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. *J. Bacteriol.* **178**:435-440.
- Fuqua, C., S. C. Winans, and E. P. Greenberg. 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol.* **50**:727-751.
- Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176**:269-275.
- Godoy, D., G. Randle, A. J. Simpson, D. M. Aanensen, T. L. Pitt, R. Kinoshita, and B. G. Spratt. 2003. Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*. *J. Clin. Microbiol.* **41**:2068-2079.
- Gotschlich, A., B. Huber, O. Geisenberger, A. Togl, A. Steidle, K. Riedel, P. Hill, B. Tummeler, P. Vandamme, B. Middleton, M. Camara, P. Williams, A. Hardman, and L. Eberl. 2001. Synthesis of multiple *N*-acylhomoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex. *Syst. Appl. Microbiol.* **24**:1-14.
- Gray, K. M., and J. R. Garey. 2001. The evolution of bacterial LuxI and LuxR quorum sensing regulators. *Microbiology* **147**:2379-2387.
- Hardman, A. M., G. S. Stewart, and P. Williams. 1998. Quorum sensing and the cell-cell communication dependent regulation of gene expression in pathogenic and non-pathogenic bacteria. *Antonie Leeuwenhoek* **74**:199-210.
- Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl. 2001. The cep quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. *Microbiology* **147**:2517-2528.
- Jeddeloh, J. A., D. L. Fritz, D. M. Waag, J. M. Hartings, and G. P. Andrews. 2003. Biodefense-driven murine model of pneumonic melioidosis. *Infect. Immun.* **71**:584-587.
- Lewenza, S., B. Conway, E. P. Greenberg, and P. A. Sokol. 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. *J. Bacteriol.* **181**:748-756.
- Lewenza, S., and P. A. Sokol. 2001. Regulation of ornibactin biosynthesis and *N*-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*. *J. Bacteriol.* **183**:2212-2218.
- Miller, W. R., L. Pannell, L. Cravitz, W. A. Tanner, and T. Rosebury. 1947. Studies on certain biological characteristics of *Malleomyces mallei* and *Malleomyces pseudomallei*. *J. Bacteriol.* **55**:115-126.
- Parsek, M. R., A. L. Schaefer, and E. P. Greenberg. 1997. Analysis of random and site-directed mutations in *rhII*, a *Pseudomonas aeruginosa* gene encoding an acylhomoserine lactone synthase. *Mol. Microbiol.* **26**:301-310.
- Roy, C. J., M. Hale, J. M. Hartings, L. Pitt, and S. Duniho. 2003. Impact of inhalation exposure modality and particle size on the respiratory deposition of ricin in BALB/c mice. *Inhal. Toxicol.* **15**:619-638.
- Rumbaugh, K. P., J. A. Griswold, and A. N. Hamood. 2000. The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa*. *Microbes Infect.* **2**:1721-1731.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg. 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J. Bacteriol.* **185**:2066-2079.
- Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, Jr., K. L. Rinehart, and S. K. Farrand. 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc. Natl. Acad. Sci. USA* **94**:6036-6041.
- Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Biotechnology* **1**:784-791.
- Smith, R. S., and B. H. Iglewski. 2003. *Pseudomonas aeruginosa* quorum-sensing systems and virulence. *Curr. Opin. Microbiol.* **6**:56-60.
- Sokol, P. A., U. Sajjan, M. B. Visser, S. Ginges, J. Forstner, and C. Kooi. 2003. The CepIR quorum-sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections. *Microbiology* **149**:3649-3658.
- Ulrich, R. L. Quorum quenching: Enzymatic disruption of *N*-acylhomoserine lactone-mediated bacterial communication in *Burkholderia thailandensis*. *Appl. Environ. Microbiol.*, in press.
- Ulrich, R. L., and D. DeShazer. 2004. Type III secretion: a virulence factor

- delivery system essential for the pathogenicity of *Burkholderia mallei*. Infect. Immun. **72**:1150–1154.
34. Ulrich, R. L., D. DeShazer, E. E. Brueggemann, H. B. Hines, P. C. Oyston, and J. A. Jeddloh. The role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. J. Med. Microbiol., in press.
35. Ulrich, R. L., H. B. Hines, N. Parthasarathy, and J. A. Jeddloh. 2004. Mutational analysis and biochemical characterization of the *Burkholderia thailandensis* DW503 quorum-sensing network. J. Bacteriol. **186**:4350–4360.
36. Valade, E., F. M. Thibault, Y. P. Gauthier, M. Palencia, M. Y. Popoff, and D. R. Vidal. 2004. The PmlI-PmlR quorum-sensing system in *Burkholderia pseudomallei* plays a key role in virulence and modulates production of the MprA protease. J. Bacteriol. **186**:2288–2294.
37. Von Bodman, S. B., W. D. Bauer, and D. L. Coplin. 2003. Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. **41**:455–482.
38. Waag, D., J. Chulay, T. Marrie, M. England, and J. Williams. 1995. Validation of an enzyme immunoassay for serodiagnosis of acute Q fever. Eur. J. Clin. Microbiol. Infect. Dis. **14**:421–427.
39. Wagner, V. E., D. Bushnell, L. Passador, A. I. Brooks, and B. H. Iglewski. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. **185**:2080–2095.

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